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Phylogenetic Analysis of *Chaenusa sensu lato* (Hymenoptera: Braconidae) using Mitochondrial NADH 1 Dehydrogenase Gene Sequences

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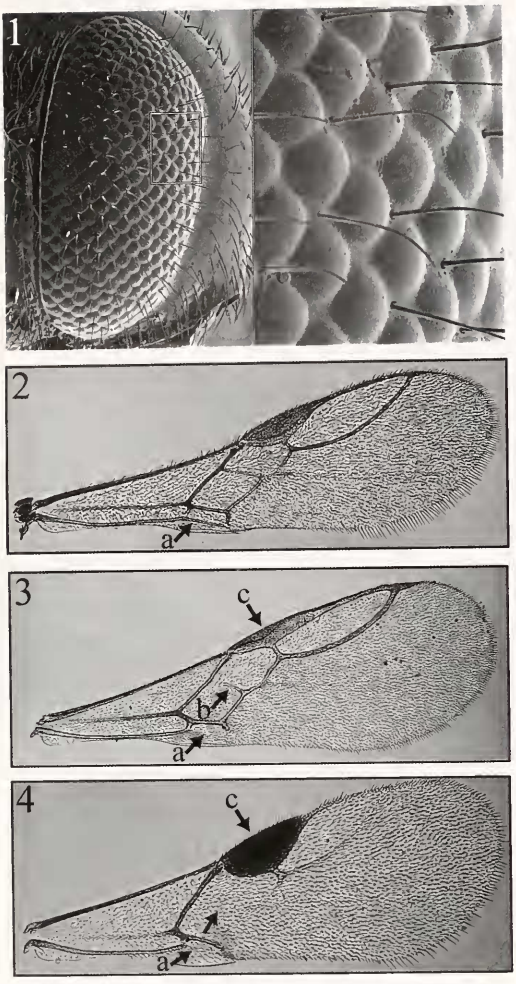
Abstract.—Alysiinae currently contains over 1,500 described species and is divided into the tribes Alysiini and Dacnusini. There is disagreement on how species should be grouped within Dacnusini, and *Chaenusa* Haliday is a prime example. *Chaenusa sensu lato* is defined by the presence of setae on the compound eyes (Griffiths 1964). Alternatively, Riegel (1950, 1982) treated *Chaenusa s.l.* as three genera, *Chaenusa sensu stricto*, *Chorebidea* Viereck, and *Chorebidella* Riegel, and differentiated the genera primarily using forewing venation and shape of the forewing stigma. Phylogenetic analyses using molecular data have not been undertaken. Therefore, we assessed the monophyly and interspecific relationships of *Chaenusa s.l.*, *Chaenusa s.s.*, *Chorebidea*, and *Chorebidella* through maximum parsimony, maximum likelihood, and Bayesian analyses using mitochondrial NADH 1 dehydrogenase gene sequences. *Chaenusa s.l.* and *Chorebidea* were not monophyletic in any of the analyses, but four of five species of *Chorebidea* always formed a clade. Further, *Chaenusa s.s.* and *Chorebidella* were monophyletic in all analyses and were always sister taxa. The results of this study largely support Riegel's (1950, 1982) treatment of *Chaenusa s.l.* as *Chaenusa s.s.*, *Chorebidea*, and *Chorebidella*. However, we suggest that *Chaenusa s.l.* be retained until additional phylogenetic analyses have been undertaken to confirm the relationships inferred in this study. In addition to the phylogenetic analyses, we discuss the morphological features relevant to Griffiths' definition of *Chaenusa s.l.* and Riegel's definition of *Chaenusa s.s.*, *Chorebidea*, and *Chorebidella*.

Alysiinae currently contains over 1,500 described species, and estimates of global richness range from 2,900 to 5,300 species (Dolphin and Quicke 2001). The monophyly of Alysiinae is firmly established based on the possession of exodont mandibles and the complete loss of the occipital carina (Griffiths 1964, Shaw and Huddleston 1991, Wharton 1997). Host records suggest that all alysiines are koinobiont endoparasitoids of cyclorrhaphous Diptera (Shaw and Huddleston 1991, Wharton and Austin 1991, Wharton 1997).

Two tribes are currently recognized in Alysiinae: Alysiini and Dacnusini. Alysiini is probably nonmonophyletic as it is defined by the presence of forewing vein r-m (a plesiomorphy). Dacnusini is considered monophyletic based on the absence of forewing vein r-m (an apomorphy) (Griffiths 1964, Shaw and Huddleston 1991, Wharton 1994) and has consistently been recognized, although at different hierarchal levels, since Förster (1862). There is widespread disagreement on how species should be grouped within Dacnusini, and *Chaenusa* Haliday is a prime example. Nixon (1943) divided dacnusines with setiferous compound eyes (Fig. 1) into two genera, *Chaenusa* and *Chorebidea* Viereck, and differentiated the genera using forewing venation and shape of the forewing stigma. Riegel (1950) established *Chorebidella* Riegel, a third genus contain-



Figs 1–4. *Chaenusa sensu lato*, *Chaenusa sensu stricto*, *Chorebidea*, and *Chorebidella*. 1, *Chorebidea americana*, setiferous compound eyes. 2, *Cha. quadriceps*, 1st subdiscal cell closed. 3, *Chorebidea saxicola*, 1st subdiscal cell open, RS+M partially present, and stigma "long". 4, *Chaenusa* sp. 3, 1st subdiscal cell open, RS+M absent, and stigma "short, wide". a = 1st subdiscal cell, b = RS+M, and c = stigma.

ing dacnusines with setiferous eyes. Like Nixon (1943) Riegel (1950) differentiated the genera primarily using forewing venation and shape of the forewing stigma. Riegel (1950) regarded all dacnusines with setiferous eyes and a closed 1st subdiscal cell as *Chaenusa* (Fig. 2); he segregated dacnusines with setiferous eyes and an open 1st subdiscal cell into *Chorebidea* or *Chorebidella*. Species with forewing vein RS+M at least partially present and a "long" stigma were considered *Chorebidea* (Fig. 3); species with RS+M absent and a "short, wide" stigma were considered *Chorebidella* (Fig. 4). Griffiths (1964) hypothesized that all dacnusines with setiferous eyes form a monophyletic group and synonymized *Chaenusa sensu stricto*, *Chorebidea*, and *Chorebidella* (i.e., *Chaenusa sensu lato*). However, Riegel (1982) disagreed with Griffiths' synonymies and continued to treat *Chaenusa sensu* Griffiths (1964) as three genera. Riegel (1982), the only comprehensive treatment of North American species of *Chaenusa s.l.*, included several new species in *Chaenusa s.s.* and *Chorebidea*, but Wharton (1997) followed *Chaenusa sensu* Griffiths (1964) rather than *Chaenusa sensu* Riegel (1982).

With 29 described species worldwide, *Chaenusa s.l.* is small relative to other dacnusine genera (e.g., over 240 species of *Chorebus* Haliday). Nearly all species are Nearctic or Palaearctic, but three species are known from Australia, and one species each is known from Madagascar and Argentina. As far as is known, flies in the ephydrid genus *Hydrellia* Robineau-Desvoidy are exclusively utilized as hosts (Griffiths 1964, Shaw and Huddleston 1991, Wharton and Austin 1991, Wharton 1997). *Hydrellia* is an important group for classical biological control of aquatic weeds. For example, *Hydrellia pakistanae* Deonier and *Hydrellia balciunasi* Bock have been imported and released for control of *Hydrilla verticillata* (L.f.) Royle in the United States. However, *Hydrellia* also contains species that are rice pests, such as *Hydrellia griseola* (Fallén) and *Hydrellia philippina* Ferino. Species of *Chaenusa s.l.* may hinder classical biological control programs as contaminants in the quarantine phase (Wharton 1997) or through parasitism (by endemics) of introduced natural enemies. Conversely, species of *Chaenusa s.l.* may be important natural enemies of pest flies (Natarajan and Mathur 1980).

Table 1. Species analyzed in this study and their respective taxonomic placements, locality data, source repositories or collectors, and GenBank accession numbers. CNG = Cimarron National Grassland, KPBS = Konza Prairie Biological Station, and SFF = Santuario de Fauna y Flora.

Species	Taxonomic Placement	Locality	Source	Accession No.
Chaenusa n. sp. 1	*Chaenusa s.s.*	Chile: Isla Chiloé, Vilupulli	UCDC	DQ917269
Chaenusa quadriceps	*Chaenusa s.s.*	Canada: ON: Ottawa	TAMU	DQ917272
Chaenusa n. sp. 2	*Chorebidea*	U.S.A.: GA: Clarke Co., Athens	TAMU	DQ917270
Chaenusa n. sp. 3	*Chorebidea*	Colombia: Boyacá, SFF Iguaque	IAVH/HIC	DQ917271
Chorebidea americana	*Chorebidea*	U.S.A.: FL: Putnam Co., Rodman Reservoir	TAMU	DQ917276
Chaenusa sp. 1	*Chorebidea*	Canada: SK: ~35 km W. Rosthern	MJY	DQ917273
Chaenusa sp. 2	*Chorebidea*	U.S.A.: SC: Lexington Co., Lexington	CNC	DQ917274
Chaenusa bergi	*Chorebidella*	U.S.A.: KS: Riley Co., KPBS	RRK/GZ	DQ917268
Chaenusa sp. 3	*Chorebidella*	India: Karnataka, Bangalore, Kumbalgodu	TAMU	DQ917275
Chorebus sp. 1	*affinis* group	U.S.A.: AZ: Santa Cruz Co., Peña Blanca Lake	RRK	DQ917277
Chorebus sp. 2	*affinis* group	U.S.A.: AZ: Santa Cruz Co., Peña Blanca Lake	RRK	DQ917278
Coelinius ferruginea	*Coelinius s.l.* (*Lepton*)	U.S.A.: KS: Riley Co., KPBS	RRK/GZ	DQ917279
Coelinius hopkinsii	*Coelinius s.l.* (*Lepton*)	U.S.A.: KS: Riley Co., KPBS	RRK/GZ	DQ917280
Dapsilarthra sp. 1	*balteata* group	U.S.A.: TX: Brazos Co., Lick Creek Park	RRK	DQ917281
Opius sp. 1	Opiinae	U.S.A.: KS: Morton Co., CNG	RRK/GZ	DQ917282

The taxonomic history discussed above illustrates that the limits of *Chaenusa* are uncertain. The monophyly of *Chaenusa s.l.*, *Chaenusa s.s.*, *Chorebidea*, and *Chorebidella* has never been assessed through phylogenetic analysis, and character systems other than morphology (e.g., DNA sequence data) have not been utilized. Resolving the taxonomic limits of *Chaenusa* and understanding the evolutionary relationships among species in the genus are important factors for predicting their potential as biological control antagonists or agents. Additionally, increased taxonomic stability facilitates revisionary work on a group. Smith et al. (1999) and Michel-Salzat and Whitfield (2004) demonstrated the utility of mitochondrial NADH 1 dehydrogenase (ND1) gene sequences for resolving evolutionary relationships among aphidiine and microgastrine braconids, respectively. Thus, the objective of this study was to assess the monophyly and interspecific relationships of *Chaenusa s.l.*, *Chaenusa s.s.*, *Chorebidea*, and *Chorebidella* using ND1 gene sequences.

MATERIALS AND METHODS

Terminology.—Terminology for mandibular teeth and external male genitalia follows Wharton (1977). Terminology for all other anatomical features, including wing cells and veins, follows Sharkey and Wharton (1997). Abbreviations for repositories are as in Evenhuis and Samuelson (2005).

Taxon sampling.—Species analyzed in this study and their respective taxonomic placements, locality data, source repositories or collectors, and GenBank accession numbers (DQ917268–DQ917282) are listed in Table 1. Specimens used for DNA isolations were acquired from repositories as indicated in Table 1 or were collected by RRK, GZ, and Matthew J. Yoder (MJY, Texas A&M University) using yellow pan traps, sweep nets, and Malaise traps. Voucher specimens for each species are deposited in the Ambrose Morell Collection for Molecular and Microbial Research at the American Museum of Natural History. With the exception of *Chaenusa pallidinervis* (Brèthes), holotypes were examined for all described alysiines dis-

cussed in this paper. The holotype of *Gyrocampa pallidinervis* Brèthes is housed in the Museo Argentina de Ciencias Naturales (MACN). The first author made multiple requests, but the MACN did not loan the holotype.

The ingroup was composed of either 13 species of Dacnusini or 13 species of Dacnusini and one species of Alysiini depending on the analysis. Nine species of *Chaenusa s.l.* were included, with *Chaenusa s.s.*, *Chorebidea*, and *Chorebidella* represented by two, five, and two species, respectively. Undescribed species were considered *Chaenusa s.s.*, *Chorebidea*, or *Chorebidella* based on forewing configuration. *Chaenusa* n. sp. 1–3 will be described in a taxonomic revision of New World *Chaenusa s.l.* (Kula in preparation). *Chaenusa* sp. 1 and 2 appear to be undescribed species but are only known from one and two individuals, respectively. Thus, RRK awaits the discovery of additional specimens before describing them. Evaluation of the literature for Old World *Chaenusa s.l.* suggests that *Chaenusa* sp. 3 is also undescribed.

Two species each from *Chorebus* and *Coelinius* Nees were also treated as ingroup taxa to test the monophyly of *Chaenusa s.l.* Species of *Chorebus* and *Coelinius* possess morphological features (i.e., eye setation, number and position of mandibular teeth, metapleural setation, metasomal compression) that suggest the potential for a close relationship with certain species of *Chaenusa s.l.* (Kula personal observation). Both species of *Chorebus* fit in the *affinis* group (Griffiths 1968), and both species of *Coelinius* fit the concept of *Lepton* Zetterstedt (=*Coelinidea* Viereck) in Griffiths (1964) (as a subgenus) and Riegel (1982) (as a genus).

A species of either *Opius* Wesmael or *Dapsilarthra* Förster was specified as the outgroup to root trees depending on the analysis. Previous phylogenetic analyses support a sister group relationship between Alysiinae and Opiinae (Quicke and van Achterberg 1990, Wharton et al. 1992, Quicke 1994, Belshaw et al. 1998, Dowton et al. 1998, Shi et al. 2005). Griffiths (1964) suggested that species of *Dapsilarthra* (Alysiini) and Dacnusini might be closely related based on parasitism of leaf-mining agromyzids. Species of *Dapsilarthra* almost exclusively attack leaf-mining agromyzids (Wharton 1984, 1997), and dacnusines that Griffiths (1964) considered morphologically plesiomorphic are parasitoids of leaf-mining agromyzids. In analyses with *Opius* sp. 1 used to root trees, *Dapsilarthra* sp. 1 was included in the ingroup to explore the monophyly of Dacnusini. *Dapsilarthra* sp. 1 was used to root trees in analyses that excluded *Opius* sp. 1.

DNA isolation, amplification, sequencing, and alignment.—Genomic DNA was isolated from individual wasps using a DNeasy® Tissue Kit (Qiagen) according to the manufacturer's protocol for insects. Most specimens were ethanol-preserved, but several were dried, pinned specimens up to 14 years old. Polymerase chain reaction (PCR) amplifications and sequencing reactions were performed using an MJ Research PTC-200 thermal cycler. A portion of the ND1 gene was amplified using PCR set up in 25 µl volume. Oligonucleotide primers (ND1F: 5′-GATAAATCAAAW-GGKGT-3′, ND1R: 5′-CAACCTTTTAGT-GATGC-3′) and the PCR program were as in Smith et al. (1999) except the annealing temperature was optimized at 47°C. PCR products were purified using a Qiaquick® PCR Purification Kit (Qiagen) according to the manufacturer's protocol. Both strands of all purified PCR products were sequenced using the PCR primers as sequencing primers. Sequencing reactions were performed in 10 µl volume using an ABI Prism® BigDye™ Terminator v3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's protocol. Sequencing reaction products were purified using spin columns filled with Sephadex® (Amersham Biosciences), dehydrated by vacuum centrifuge, and sent to the DNA Sequenc-

ing & Synthesis Facility at Iowa State University for gel runs on an ABI Prism® 3700 DNA Analyzer (Applied Biosystems). Sequences generated from the forward and reverse primers were aligned and edited in Sequencher™ 4.1.2 (Gene Codes Corporation) to acquire a consensus sequence for each species. Consensus sequences were manually aligned in SeqPup 0.6 (Gilbert 1996) to produce a DNA sequence data matrix. The DNA data matrix was translated to construct an amino acid (AA) sequence data matrix using the *Drosophila* Fallén mtDNA genetic code in MacClade 4.06 (Maddison and Maddison 2003).

DNA and AA sequence characteristics and phylogenetic analysis.—The number of constant, variable parsimony uninformative, and parsimony informative characters were determined using PAUP* 4.0b10 (Swofford 2002), as were mean base frequencies. PAUP* 4.0b10 was also used to test for significant heterogeneity of base frequencies across taxa; base frequencies were considered significantly heterogeneous if $P \leq 0.05$.

Maximum parsimony (MP) and maximum likelihood (ML) analyses were performed using PAUP* 4.0b10. Maximum parsimony analyses were conducted for the DNA and AA data matrices using the branch and bound algorithm. Modeltest 3.06 (Posada and Crandall 1998) was used to determine the model of molecular evolution that best fit the data, and subsequently, ML analyses were conducted for the DNA data matrix using the heuristic search option with stepwise addition, 100 random addition sequence replicates, and tree bisection-reconnection (TBR) branch swapping. If the Hierarchical Likelihood Ratio Test (hLRT) and the Akaike Information Criterion (AIC) in Modeltest selected different models, ML analyses were performed using each model. Support for individual clades was assessed via bootstrap analyses. For MP 1,000 pseudoreplicates with the branch and bound algorithm were used. For ML 100 pseudoreplicates using the heuristic search option with stepwise addition, 50 random addition sequence replicates, and TBR branch swapping were used.

Bayesian analyses were performed using MrBayes 3.0b4 (Huelsenbeck and Ronquist 2001). Modeltest 3.06 was used to determine the model of molecular evolution that best fit the data, and subsequently, Bayesian analyses were performed for the DNA data matrix. The data matrix was partitioned by codon position (i.e., 1st, 2nd, 3rd), and among-site rate variation was set (as a prior) to allow variable rates across partitions. The model of nucleotide substitution and among-site rate variation was set as determined using Modeltest. The following model parameters were unlinked across the partitions: substitution rates of GTR model, character state frequencies, gamma shape parameter, and proportion of invariable sites. Each run consisted of 1,000,000 generations with a random starting tree and sample frequency of every 100 generations. The burnin was determined by constructing an XY scatter plot (i.e., generation $\times$ log likelihood value) using Microsoft® Excel to determine the number of generations until log likelihood values stabilized. Trees sampled prior to the generation at which log likelihood values stabilized were not included in the consensus tree. A 50% majority-rule consensus of the retained trees, showing the frequency of all observed bipartitions (i.e., posterior probabilities), was constructed using PAUP* 4.0b10.

Maximum parsimony analyses with *Chaenusa s.l.* constrained as monophyletic were also performed for the DNA data matrix. The search parameters were the same as for unconstrained MP analyses as discussed above. Most parsimonious trees (MPTs) from unconstrained and constrained analyses were compared statistically using the "Compare-2" permutation test (Faith 1991) in PAUP* 4.0b10. Under MP each of 10,000 random matrices (with

Table 2. Number of constant (C), variable parsimony uninformative (VPU), and parsimony informative (PI) characters for all nucleotide (nuc) and amino acid (AA) sites and positions (Pos) 1, 2, and 3 with *Opius* sp. 1 excluded (excl) and included (incl).

Dataset	C	VPU	PI
All nuc sites (*Opius* sp. 1 excl)	241	66	123
All nuc sites (*Opius* sp. 1 incl)	234	60	136
Pos 1 (*Opius* sp. 1 excl)	83	26	34
Pos 1 (*Opius* sp. 1 incl)	81	25	37
Pos 2 (*Opius* sp. 1 excl)	119	7	17
Pos 2 (*Opius* sp. 1 incl)	116	8	19
Pos 3 (*Opius* sp. 1 excl)	39	33	72
Pos 3 (*Opius* sp. 1 incl)	37	27	80
All AA sites (*Opius* sp. 1 excl)	79	24	40
All AA sites (*Opius* sp. 1 incl)	74	26	43

all taxa randomized) were analyzed using the heuristic search option with stepwise addition, 500 random addition sequence replicates, and TBR branch swapping. The length difference between two trees (i.e., alternative hypotheses of relationships) was considered significant if $P \leq 0.05$.

RESULTS

DNA and AA sequence characteristics.—After sequence editing the aligned DNA data matrix was 430 bp and included no gaps. The DNA data matrix translated to an AA data matrix of 143 AAs. The number of constant, variable parsimony uninformative, and parsimony informative characters for all sites and positions 1, 2, and 3 with *Opius* sp. 1 excluded and included are reported in Table 2.

Evaluation of the mean base frequencies revealed a high A+T nucleotide bias, particularly in the first and third positions (Table 3). However, significant heterogeneity of base frequencies across taxa was detected only for position 3 when *Opius* sp. 1 was included (Table 4). High A+T nucleotide bias and less constrained nucleotide change relative to positions 1 and 2 may cause a high level of homoplasy in position 3 of insect mitochondrial protein-coding genes. Therefore, MP and bootstrap analyses were performed, as described above, with *Opius* sp. 1 included and position 3 excluded.

In Modeltest the hLRT selected the TIM model with a proportion of invariable sites and gamma distributed rate variation among sites; the AIC selected the TrN model with a proportion of invariable sites and gamma distributed rate variation among sites. Maximum likelihood analyses using each model resulted in trees with identical topologies, and the results of analyses using the TrN model are presented below.

Phylogenetic analysis.—Maximum parsimony analysis of the DNA data matrix with *Opius* sp. 1 excluded resulted in two MPTs (tree length = 387 steps, consistency index excluding uninformative characters (CI) = 0.5609, retention index (RI) = 0.5959) (Fig. 5). The trees differed only in the placement of *Chaenusa* n. sp. 3 as either sister to *Chorebus* sp. 1 or sister to the rest of the ingroup. *Chaenusa s.l.* was not mono-

Table 3. Mean base frequencies for all sites and positions (Pos) 1, 2, and 3 with *Opius* sp. 1 excluded (excl) and included (incl).

Dataset	A	C	G	T
All sites (*Opius* sp. 1 excl)	0.35626	0.10279	0.07695	0.46401
All sites (*Opius* sp. 1 incl)	0.35709	0.10381	0.07671	0.46239
Pos 1 (*Opius* sp. 1 excl)	0.35317	0.07908	0.09507	0.47267
Pos 1 (*Opius* sp. 1 incl)	0.35514	0.07901	0.09441	0.47144
Pos 2 (*Opius* sp. 1 excl)	0.20829	0.18746	0.11588	0.48836
Pos 2 (*Opius* sp. 1 incl)	0.20812	0.18726	0.11619	0.48843
Pos 3 (*Opius* sp. 1 excl)	0.50735	0.04165	0.02022	0.43078
Pos 3 (*Opius* sp. 1 incl)	0.50803	0.04496	0.01983	0.42719

Table 4. Results of tests for significant heterogeneity of base frequencies across taxa for all sites and positions (Pos) 1, 2, and 3 with *Opius* sp. 1 excluded (excl) and included (incl).

Dataset	χ^2	*P*
All sites (*Opius* sp. 1 excl)	17.509269	0.99882241
All sites (*Opius* sp. 1 incl)	19.096701	0.99908575
Pos 1 (*Opius* sp. 1 excl)	12.619144	0.99998081
Pos 1 (*Opius* sp. 1 incl)	13.202722	0.99999385
Pos 2 (*Opius* sp. 1 excl)	2.302238	1.00000000
Pos 2 (*Opius* sp. 1 incl)	2.338632	1.00000000
Pos 3 (*Opius* sp. 1 excl)	53.386385	0.06219822
Pos 3 (*Opius* sp. 1 incl)	60.168328	0.03417160

phyletic. In both trees the *Coelinius* clade was sister to the clade formed by four of the five species of *Chorebidea* included in the analysis. Further, *Chaenusa* n. sp. 3 either formed a clade with *Chorebus* sp. 1 and *Chorebus* sp. 2 or was sister to the rest of the ingroup. *Chorebidea* was not monophyletic, although four of five species of *Chorebidea* included in the analysis formed a clade with 94% bootstrap support. *Chorebus* was monophyletic in one tree, but bootstrap support was <50%. *Chaenusa s.s.*, *Chorebidella*, and *Coelinius* were monophyletic with 97%, 100%, and 75% bootstrap support, respectively. Bootstrap support for the relationships among these clades was <50% except for the sister group relationship between *Chaenusa s.s.* and *Chorebidella* (99% bootstrap support).

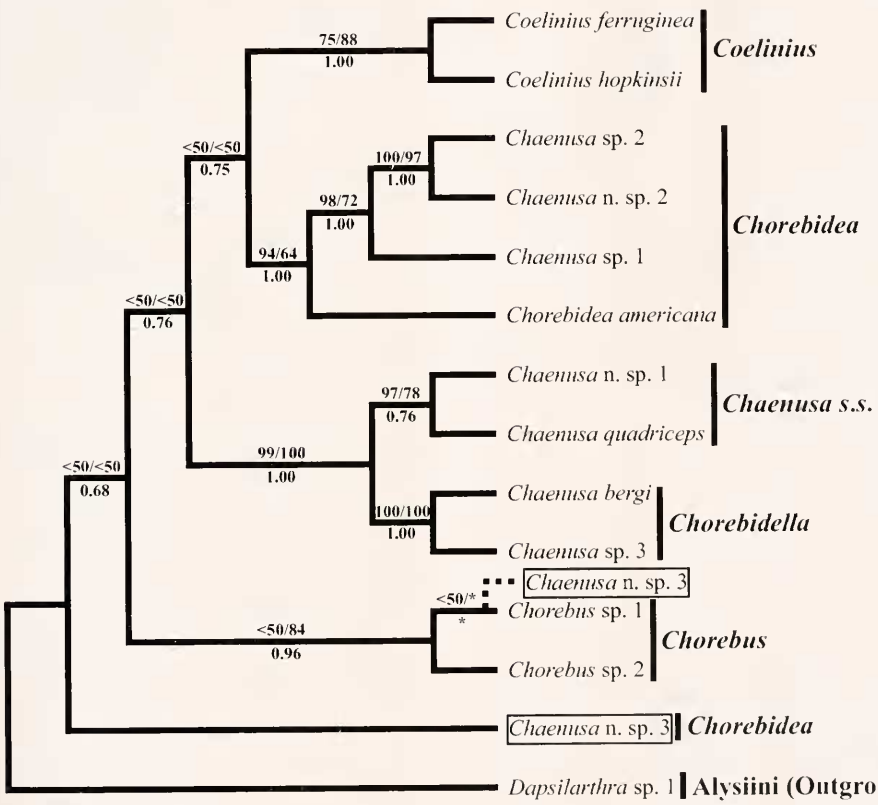


Fig. 5. Composite cladogram of two most parsimonious trees resulting from maximum parsimony analysis of the DNA data matrix with *Opius* sp. 1 excluded. Dashed line indicates alternative placement of *Chaenusa* n. sp. 3. Maximum parsimony bootstrap values are above branches and left of slashes. Where clades were recovered in maximum likelihood (ML) analysis with *Opius* sp. 1 excluded, bootstrap values are above branches and right of slashes. Where clades were recovered in Bayesian analysis with *Opius* sp. 1 excluded, posterior probabilities are below branches. Asterisks above and below branches indicate clades not recovered in ML and Bayesian analysis, respectively.

Table 5. Bootstrap support and posterior probabilities for groups within Dacnusini recovered through maximum parsimony (MP) and Bayesian analyses with *Opius* sp. 1 included. Maximum parsimony analyses were conducted with position (Pos) 3 included (incl) and excluded (excl). nr = groups not recovered.

Group	MP (Pos 3 incl)	MP (Pos 3 excl)	Bayesian
Chaenusa s.s.	97	100	0.77
Chorebidella	100	99	1.00
4 of 5 *Chorebidea*	98	85	1.00
Chaenusa s.s. + *Chorebidella*	99	81	1.00
Chorebus	nr	nr	0.88
Coelinius	67	67	1.00
Chorebus sp. 1 + *Chaenusa* n. sp. 3	59	<50	nr
Chorebus + *Chaenusa* n. sp. 3	62	<50	0.84

Maximum likelihood and Bayesian analyses of the DNA data matrix with *Opius* sp. 1 excluded resulted in a most likely tree (-Ln likelihood value = 2246.66073) and a 50% majority-rule consensus tree, respectively, with topologies identical to the MP tree with *Chaenusa* n. sp. 3 sister to the rest of the ingroup (Fig. 5). For the Bayesian consensus tree, the burnin was the first 50 trees. Bootstrap support for ML and posterior probabilities for Bayesian are reported in Fig. 5.

Maximum parsimony analysis with *Opius* sp. 1 included resulted in a single MPT (tree length = 435 steps, CI = 0.5474, RI = 0.5729) (tree not shown). Dacnusini was monophyletic with 78% bootstrap support. The relationships among dacnusines were identical to the MP tree with *Opius* sp. 1 excluded and *Chaenusa* n. sp. 3 sister to *Chorebus* sp. 1 (Fig. 5). Analysis with position 3 excluded resulted in a single MPT (tree length = 177, CI = 0.5755, RI = 0.6118) (tree not shown) with a topology identical to the tree with position 3 included. Dacnusini was monophyletic, but bootstrap support was <50%. Bootstrap support for groups within Dacnusini for analyses with position 3 included and excluded are presented in Table 5.

Bayesian analysis of the DNA data matrix with *Opius* sp. 1 included resulted in a 50% majority-rule consensus tree (tree not shown) with a topology nearly identical to the MP tree with *Opius* sp. 1 excluded and *Chaenusa* n. sp. 3 sister to *Chorebus* sp. 1 (Fig. 5). The burnin was the first 70 trees. In terms of the relationships among dacnusines, the only differences between the trees were (1) *Chorebus* was monophyletic with *Chaenusa* n. sp. 3 sister to the *Chorebus* clade and (2) the clade containing all dacnusines except *Chaenusa* n. sp. 3, *Chorebus* sp. 1, and *Chorebus* sp. 2 was not recovered. Dacnusini was monophyletic with a posterior probability of 0.99. Posterior probabilities for groups within Dacnusini are presented in Table 5.

Maximum likelihood analysis of the DNA data matrix with *Opius* sp. 1 included resulted in a most likely tree (−Ln likelihood value = 2437.12877) with a topology considerably different than trees from all other analyses (Fig. 6). Dacnusini was not monophyletic. Rather, *Dapsilarthra* sp. 1 was sister to *Chaenusa* n. sp. 3, but bootstrap support for this relationship was <50%. *Chaenusa s.l.* was not monophyletic. *Chorebidea* was not monophyletic, although four of five species of *Chorebidea* included in the analysis formed a clade with 67% bootstrap support. *Chaenusa s.s.*, *Chorebidella*, *Chorebus*, and *Coelinius* were monophyletic with 75%, 99%, 80%, and 71% bootstrap support, respectively. Bootstrap support for the relationships among these clades was <50% except for the sister group relationship between *Chaenusa s.s.* and *Chorebidella* (98% bootstrap support).

Maximum parsimony analysis with *Opius* sp. 1 excluded and *Chaenusa s.l.* constrained as monophyletic resulted in two MPTs (tree length = 393 steps, CI = 0.5503, RI = 0.5782) (trees not shown) six steps longer than the MPTs from the unconstrained analysis. The "Compare-2" test revealed that the two MPTs from the

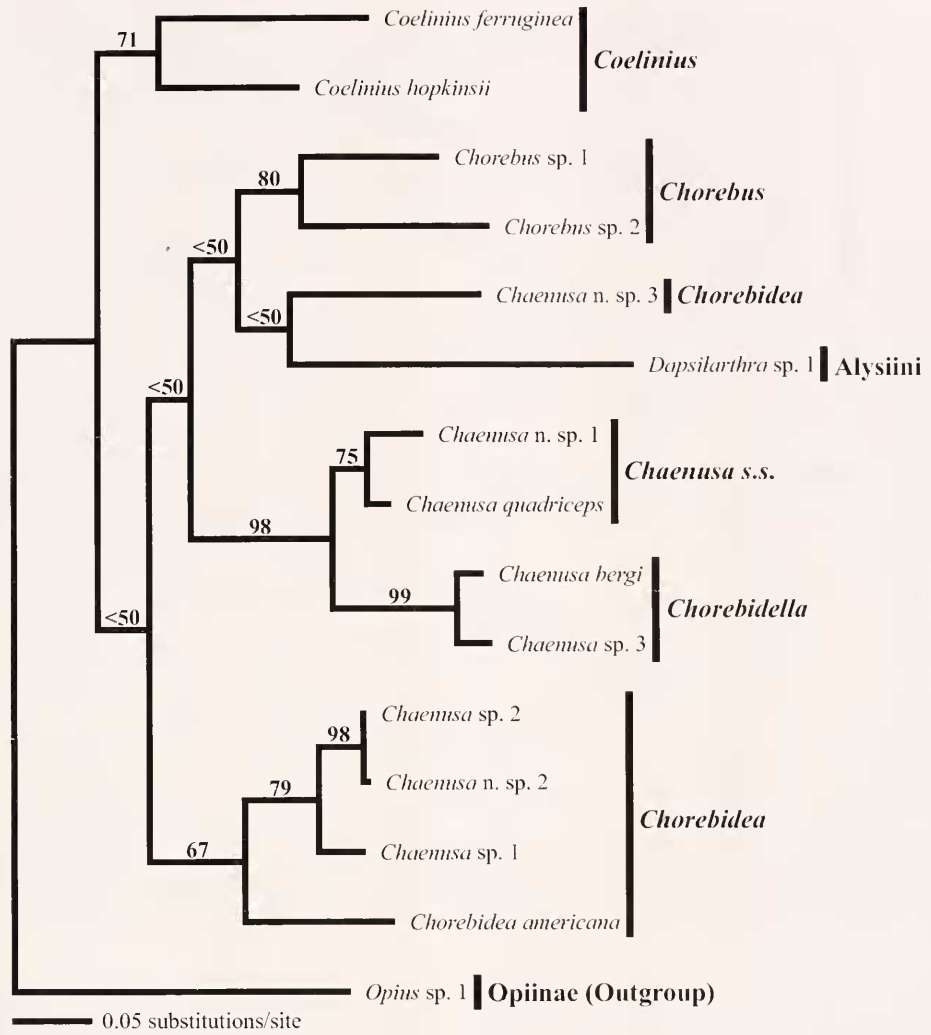


Fig. 6. Phylogram resulting from maximum likelihood analysis of the DNA data matrix with *Opius* sp. 1 included. Bootstrap values are above branches.

unconstrained analysis are not significantly shorter than either of the two MPTs from the constrained analysis (P = 0.125100, 0.127600, 0.162700, 0.165300). Maximum parsimony analysis with *Opius* sp. 1 included and *Chaenusa s.l.* constrained as monophyletic resulted in one MPT (tree length = 444 steps, CI = 0.5344, RI = 0.5499) (tree not shown) nine steps longer than the MPT from the unconstrained analysis. The "Compare-2" test revealed that the MPT from the unconstrained analysis is significantly shorter than the MPT from the constrained analysis (P = 0.031300).

Maximum parsimony analysis of the AA data matrix with *Opius* sp. 1 included resulted in two MPTs (tree length = 161 steps, CI = 0.7087, RI = 0.7176). Dacnusini was not monophyletic in the strict consensus of the two MPTs (Fig. 7). Rather, *Dapsilarthra* sp. 1 was sister to *Chaenusa* n. sp. 3, but bootstrap support for this relationship was <50%. *Chaenusa s.l.* was not monophyletic. *Chorebidea* was not monophyletic, although four of five species of

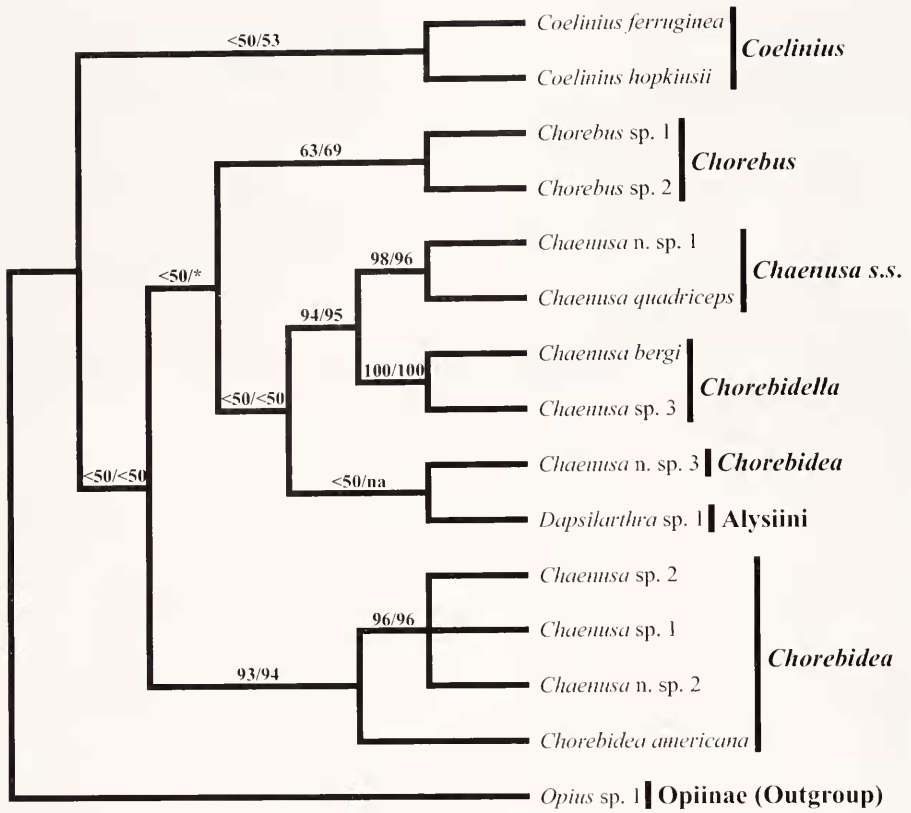


Fig. 7. Strict consensus of two most parsimonious trees resulting from maximum parsimony (MP) analysis of the amino acid data matrix with *Opius* sp. 1 included. Maximum parsimony bootstrap values are above branches and left of slashes. Where clades were recovered in MP analysis with *Opius* sp. 1 excluded, bootstrap values are above branches and right of slashes. The asterisk indicates a clade not recovered in MP analysis with *Opius* sp. 1 excluded. na = not applicable.

Chorebidea included in the analysis formed a clade with 93% bootstrap support. *Chaenusa s.s.*, *Chorebidella*, and *Chorebus* were monophyletic with bootstrap support of 98%, 100%, and 63%, respectively. *Coelinius* was monophyletic, but bootstrap support was <50%. Bootstrap support for the relationships among these clades was <50% except for the sister group relationship between *Chaenusa s.s.* and *Chorebidella* (94% bootstrap support).

Maximum parsimony analysis of the AA data matrix with *Opius* sp. 1 excluded resulted in six MPTs (tree length = 142 steps, CI = 0.7273, RI = 0.7479). In terms of the relationships among dacnusines, the strict consensus of the six MPTs (tree not shown) was identical to the strict consensus tree in Fig. 7 except the monophyly of *Chaenusa s.l.* was unresolved (see asterisk in Fig. 7). Bootstrap support is reported in Fig. 7.

DISCUSSION

DNA sequence characteristics.—Characteristics of the ND1 DNA sequences in this study are consistent with ND1 DNA sequences of other braconids (e.g., Smith and Kambhampati 1999, Smith et al. 1999, Michel-Salzat and Whitfield 2004). As in the aforementioned studies, the sequenced fragments in this study (including *Opius* sp. 1) are biased towards adenine (35.7%) and thymine (46.2%), particularly in the first (82.7%) and third (93.5%) positions. Significant heterogeneity of base frequen-

cies was detected for position 3 when *Opius* sp. 1 was included. As mentioned in Michel-Salzat and Whitfield (2004), the A+T nucleotide bias observed for insect mitochondrial DNA could influence the level of homoplasy, particularly in the first and third positions. However, 27.2% and 58.8% of the parsimony informative characters in the DNA data matrix with *Opius* sp. 1 included are in the first and third positions, respectively. Therefore, for most analyses all positions were considered and were not differentially weighted. We performed MP and bootstrap analyses with *Opius* sp. 1 included and position 3 excluded to examine the influence of position 3 on tree topology and branch support. The exclusion of position 3 had no influence on tree topology but resulted in lower bootstrap support for several clades. Conversely, there was a slight increase in CI and RI values when position 3 was excluded. This suggests that position 3 contains phylogenetic information that supports several clades but also increases the level of homoplasy in the data matrix.

Tribe Dacnusini.—Griffiths (1964) and Wharton (1994) suggested that Dacnusini is monophyletic based on the absence of forewing vein r-m. Further, Dacnusini is homogeneous in terms of host utilization; the tribe exclusively contains parasitoids of plant-mining flies, particularly parasitoids of leaf- and stem-mining agromyzids, chloropids, and ephydrids (Wharton 1997). Maximum parsimony, ML, and Bayesian analyses were conducted with *Dapsilarthra* sp. 1 included in the ingroup to explore the monophyly of Dacnusini. In MP and Bayesian analyses of the DNA data matrix, Dacnusini was monophyletic with 78% bootstrap support and a posterior probability of 0.99, respectively. However, neither ML analysis of the DNA data matrix nor MP analysis of the AA data matrix recovered Dacnusini. Rather, *Dapsilarthra* sp. 1 was always sister to *Chaenusa* n. sp. 3, but bootstrap support for this relationship was <50%. In MP analysis of the AA data matrix with *Chaenusa* n. sp. 3 excluded, Dacnusini was monophyletic in two of six MPTs, but bootstrap support was <50% (results not presented). Dacnusini was not monophyletic in ML analysis of the DNA data matrix with *Chaenusa* n. sp. 3 excluded (results not presented). Thus, ND1 DNA sequences and the absence of forewing vein r-m largely, but not conclusively, support the monophyly of Dacnusini. Exclusive utilization of plant-mining flies as hosts, particularly leaf- and stem-mining agromyzids, chloropids, and ephydrids (i.e., biological homogeneity), provides further indication that Dacnusini is monophyletic. However, more extensive taxon sampling and the use of additional markers more conserved than ND1 are needed to confirm the monophyly of Dacnusini and resolve the more ancient divergences within the tribe.

Genus Chaenusa sensu lato.—*Chaenusa s.l.* was not monophyletic in any of the analyses. Rather, the results indicate that certain species of *Chaenusa s.l.* are more closely related to species of *Chorebus* and *Coelinius* than they are to other species of *Chaenusa s.l.* This result is not surprising for several reasons. Certain species of *Chaenusa s.l.* possess morphological features that suggest the potential for a close relationship with species of *Chorebus* and *Coelinius*. As is observed for species of the *Chorebus affinis* group (Griffiths 1968), several species of *Chaenusa s.l.* have four-toothed mandibles with three major teeth and one small tooth along the ventral margin of elongate tooth 2. In this study *Chaenusa* n. sp. 3, *Chaenusa* n. sp. 1, and *Cha. quadriceps* (Ashmead) exhibit this condition, as do four described (i.e., *Chaenusa anticostae* Riegel, *Chaenusa californica* Riegel, *Chaenusa illinae* Riegel, *Chaenusa rossi* Riegel) and two undescribed Nearctic species of *Chaenusa s.l.* not included in this study (Kula unpublished). Further, the metapleural setation of *Chaenusa* n. sp. 3 is nearly oriented in a rosette surrounding a raised swelling, a character state used to

define *Chorebus*. *Chaenusa* n. sp. 3 forms a clade with *Chorebus* in certain MP and Bayesian analyses, and it is possible that *Chaenusa* n. sp. 3 is a species of *Chorebus* with setiferous eyes.

A character state in females of *Coelinius* is lateral compression of the metasoma. Females of *Chorebidea americana* Riegel, *Chorebidea bessae* Riegel, *Chorebidea mcclurei* Riegel, *Cha. rossi*, *Chorebidea saxicola* Riegel, and one undescribed Nearctic species of *Chaenusa s.l.* have a laterally compressed metasoma (Kula unpublished). In this study only *Chorebidea americana* clearly exhibits this condition. Further, *Coelinius* is partially defined on the possession of four-toothed mandibles with three major teeth and one small tooth between tooth 1 and 2. In this study *Chaenusa* sp. 2, *Chaenusa* n. sp. 2, and *Chaenusa* sp. 1 exhibit this condition, and it also occurs in an undescribed Nearctic species of *Chaenusa s.l.* not included in this study (Kula unpublished).

Griffiths (1964) proposed that among dacnusines setiferous eyes is unique to species of *Chaenusa s.l.* and is a synapomorphy that defines *Chaenusa s.l.* However, dacnusines in genera other than *Chaenusa s.l.* have setiferous eyes. New World species of *Chorebus* (47 morphospecies), *Coelinius* (19 morphospecies), *Coloneura* Förster (two morphospecies), *Dacnusa* Haliday (18 morphospecies), *Epimicta* Förster (two morphospecies), *Exotela* Förster (14 morphospecies), *Laotris* Nixon (six specimens), and *Synelix* Förster (one morphospecies) all have setiferous eyes. Only New World species of *Symphya* Förster (13 morphospecies) have glabrous eyes (Kula unpublished). Character states other than setiferous eyes clearly place the aforementioned species in their respective genera. In most cases the setae are straight and are so minute that they could easily escape detection using a stereomicroscope at 120× magnification (i.e., usually shorter than a facet width). For species of *Chaenusa s.l.*, at least some setae on the eyes are conspicuously longer than a facet width and are curved. However, 8.5% of the *Chorebus* and 5.3% of the *Coelinius* morphospecies examined have curved setae on the eyes longer than a facet width. Thus, the mere presence of setae on the eyes cannot be regarded as a synapomorphy that defines *Chaenusa s.l.*

Genus Chaenusa sensu stricto.—Chaenusa s.s. was monophyletic in all analyses, and branch support was moderate to strong. *Chaenusa s.s.* should be more extensively sampled in future phylogenetic analyses to provide a more robust assessment of monophyly. Six of the 11 described New World species of *Chaenusa s.l.* fit in *Chaenusa s.s.* (i.e., *Cha. anticostae*, *Cha. californica*, *Cha. illinae*, *Cha. pallidinervis*, *Cha. quadriceps*, *Cha. rossi*). However, all except *Cha. quadriceps* are only known from the holotype. Thus, a very small number of New World specimens of *Chaenusa s.s.* are available for DNA sequencing. Extensive collecting will be needed to increase the representation of New World *Chaenusa s.s.* in future phylogenetic analyses. The most successful methods for collecting specimens of *Chaenusa s.l.* are yellow pan traps placed along the shore of permanent lakes, ponds, and streams and sweeping within and along the edge of aquatic habitats.

Riegel (1950, 1982) defined *Chaenusa s.s.* using the following features: (1) 1st subdiscal cell closed, (2) stigma "short, wide", and (3) labial palpi four-segmented. Both species of *Chaenusa s.s.* included in this study have the 1st subdiscal cell closed, a relatively broad stigma, and three- or four-segmented labial palpi. The length of the distal palpomere in specimens with three-segmented labial palpi is approximately the combined length of palpomeres 3 and 4 in specimens with four-segmented labial palpi. Further, examination with a scanning electron microscope revealed that the distinction between palpomeres 3 and 4 is extremely weak in some specimens of *Chaenusa* n. sp. 1, *Cha. quadriceps*, and an

undescribed Nearctic species that fits *Chaenusa s.s.* Thus, it appears that three-segmented labial palpi in *Chaenusa* n. sp. 1 and *Cha. quadriceps* resulted from the fusion of palpomeres 3 and 4 or the division of palpomere 3 into two palpomeres.

Genus Chorebidea.—*Chorebidea* was not monophyletic in any of the analyses. However, four of five species of *Chorebidea* included in this study formed a clade in all analyses, and branch support was weak to strong. Riegel (1950, 1982) defined *Chorebidea* using the following features: (1) 1st subdiscal cell open, (2) forewing vein RS+M at least partially present, (3) stigma "long", (4) labial palpi three-segmented, and (5) gonoforceps "stocking-shaped in lateral view". All species of *Chorebidea* included in this study have an open 1st subdiscal cell through the partial or complete absence of forewing veins 2-1A and 2cu-a, and forewing vein RS+M is at least partially present. Both features exhibit some degree of intraspecific variation. The 1st subdiscal cell is rarely (3.1%, one of 32 specimens examined) closed in *Chaenusa* n. sp. 3, and although forewing vein RS+M is present for all species, it may vary from complete and tubular to minutely present posteriorly. Riegel (1950, 1982) included a "long" stigma in his concept of *Chorebidea*, but *Chorebidea americana* and *Chorebidea bessae* have a relatively broad stigma. The stigma is relatively long for *Chaenusa* sp. 2, *Chaenusa* n. sp. 2, *Chaenusa* sp. 1, and *Chaenusa* n. sp. 3 but is relatively broad for *Chorebidea americana*. *Chaenusa* sp. 2, *Chaenusa* n. sp. 2, *Chaenusa* sp. 1, and *Chorebidea americana* have three-segmented labial palpi, but the labial palpi are four-segmented for *Chaenusa* n. sp. 3. Lastly, *Chorebidea americana* has "stocking-shaped" gonoforceps, but *Chaenusa* sp. 2, *Chaenusa* n. sp. 2, and *Chaenusa* sp. 1 have gonoforceps that gradually narrow proximally to distally and are roughly triangular-shaped. *Chaenusa* n. sp. 3 has roughly rectangular-shaped gonoforceps that are truncate distally.

Genus Chorebidella.—*Chorebidella* was monophyletic in all analyses, and branch support was strong. *Chorebidella* should be more extensively sampled in future phylogenetic analyses to provide a more robust assessment of monophyly. Only one of the 11 described New World species of *Chaenusa s.l.* fits in *Chorebidella* (i.e., *Chaenusa bergi* (Riegel)). We acquired two Old World species in addition to *Cha. bergi* but only had permission to use one for DNA sequencing. As for *Chaenusa s.s.* extensive collecting will be needed to increase the representation of New World *Chorebidella* in future phylogenetic analyses.

Riegel (1950, 1982) defined *Chorebidella* using the following features: (1) 1st subdiscal cell open, (2) forewing vein RS+M absent, (3) stigma "short, wide", (4) labial palpi three-segmented, and (5) gonoforceps "not stocking-shaped in lateral view". Both species of *Chorebidella* included in this study have the 1st subdiscal cell open through the partial or complete absence of forewing veins 2-1A and 2cu-a, forewing vein RS+M absent, a relatively broad stigma, and gonoforceps that gradually narrow proximally to distally and are roughly triangular-shaped. *Chaenusa bergi* has three-segmented labial palpi, but *Chaenusa* sp. 3 has two-segmented labial palpi. Two-segmented labial palpi have not been recorded for any species of *Chaenusa s.l.*

CONCLUSIONS

The results of this study indicate that *Chaenusa s.l.* is not monophyletic, but *Chaenusa s.s.* and *Chorebidella* are monophyletic groups with moderate to strong support. *Chorebidea* was not monophyletic in any of the analyses, but four of five species of *Chorebidea* included in this study formed a clade in all analyses. The species of *Chorebidea* that did not form a clade with the other species of *Chorebidea* (i.e., *Chaenusa* n. sp. 3) exhibits morphological character states observed for species of *Chorebus*. Further, *Chaenusa* n. sp. 3 forms a clade with *Chorebus* in certain MP and

Bayesian analyses, and this suggests that *Chaenusa* n. sp. 3 may actually be a species of *Chorebus* with long curved setae on the eyes.

Phylogenetic analyses using ND1 gene sequences largely support Riegel's (1950, 1982) treatment of *Chaenusa s.l.* as *Chaenusa s.s.*, *Chorebidea*, and *Chorebidella*. However, we suggest that *Chaenusa s.l.* be retained until phylogenetic analyses with nuclear markers, morphology, and greater taxon sampling have been undertaken to confirm the relationships inferred in this study.

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